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Protonation Behavior of Histidine during HSF1 Activation by Physiological Acidification

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ABSTRACT

The expression of eukaryotic molecular chaperones (heat shock proteins, HSPs) is triggered in response to a wide range of environmental stresses, including: heat shock, hydrogen peroxide, heavy metal, low-pH, or virus infection. Biochemical and genetic studies have clearly shown the fundamental roles of heat shock factor 1 (HSF1) in stress-inducible HSP gene expression, resistance to stress-induced cell death, carcinogenesis, and other biological phenomena. Previous studies show that acidic pH changes within the physiological range directly activate the HSF1 function in vitro. However, the detailed mechanism is unclear. Though computational pKa-predications of the amino acid side-chain, acidic-pH induced protonation of a histidine residue was found to be most-likely involved in this process. The histidine 83 (His83) residue, which could be protonated by mild decrease in pH, causes mild acidic-induced HSF1 activation (including in-vitro trimerization, DNA binding, in-vivo nuclear accumulation, and HSPs expression). His83, which is located in the loop region of the HSF1 DNA binding domain, was suggested to enhance the intermolecular force with Arginine 79, which helps HSF1 form a DNA-binding competent. Therefore, low-pH-induced activation of HSF1 by the protonation of histidine can help us better to understand the HSF1 mechanism and develop more therapeutic applications (particularly in cancer therapy). J. Cell. Biochem. 116: 977–984, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: HEAT SHOCK FACTOR 1 (HSF1); PROTONATION; HISTIDINE; ACIDIFICATION

All organisms respond to environmental stress in a process, which has been historically known as the heat shock response (HSR), by activating genes that encode molecular chaperones and other cytoprotective proteins (HSPs) [Akerfelt et al., 2010; Anckar and Sistonen, 2011]. A compromised HSR has been associated with normal aging and numerous age-related diseases [Morimoto, 2008; Calderwood et al., 2009]. Senescent cells and neurons are particularly vulnerable to stress due to the reduced levels of HSPs needed to prevent cytotoxic protein misfolding and aggregation. In contrast, the cytoprotective activities of HSPs allow the accumulation of oncogenic alterations in DNA and in proteins, which could lead to cell transformation and to resistance of the conventional therapy [Calderwood et al., 2006; Whitesell and Lindquist, 2009].

The HSR is regulated predominantly at the transcriptional level by a family of heat shock factors (HSFs). HSF1 is a master regulator of the HSR in mammalian cells. The in-vitro translated or purified HSF1 could be activated directly by low pH, heavy metal, H_2O_2, or salicylate [Mosser et al., 1990; Zhong et al., 1999]. Under these stressors, HSF1 monomers can trimerize rapidly and acquire the ability to bind to the heat shock element (HSE) in the promoter of the heat shock genes [Wu, 1995]. The transcriptional competence of HSF1 is modulated by phosphorylation, sumoylation, and acetylation [Hietakangas et al., 2003; Anckar et al., 2006; Xu et al., 2008; Westerheide et al., 2009]. Recent studies demonstrated a new view, unlike the protective roles in HSR, HSF1 plays a highly malignant (but not a protective) role in carcinogenesis (such as malignant transformation, cancer cell survival, and proliferation in human cancer cell lines and mouse models) [Dai et al., 2007; Mendillo et al., 2012]. Therefore, a better understanding of the HSF1 mechanism (especially in cancer) can facilitate the development of more therapeutic applications.

To our knowledge, tumor tissues could excessively uptake nutrients from the blood (such as glucose and oxygen), and eventually release a mass of acidic metabolites (lactic acid). Cancer cells are exposed to an oxygen-deficient (hypoxic) and acidic environment which allows them to flourish within tumor tissues

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[Dang, 2012]. High level of the lactic-acid accumulation could directly decrease the internal pH (pH_i) within the solid tumor tissues from 7.3 to 6.5. Especially in some terminal cancer cases, the pH_i of tumors was ghastly down to 6.0 or even 5.7 [Hirschhaeuser et al., 2011; Dang, 2012]. The body pH of terminal cancer patients is over 100 times more acidic than that of normal healthy people. Meanwhile, many cancer-related researches found a phenomenon that the inducible chaperones HSP70 (member of the HSP70 superfamily), HSP40 family, and HSP27 (member of the small HSP family), which are mainly regulated by heat shock factor 1 (HSF1), exhibit the elevated expression levels [Ciocca and Calderwood, 2005]. Coincidentally, a pH_i-drop phenomenon (pH_i was down to 6.0) was also found in the heat-induced animal cells, along with the activation of HSF1 and over-expression of HSPs [Drummond et al., 1986; Zhong et al., 1999; Coss et al., 2004]. All above evidences strongly suggest a potential relationship between the intracellular acidification and HSF1 activation. In this study, we showed that the low pH in the physiological range (pH ~6.0) can directly induce HSF1 trimerization and DNA-binding in vitro and in vivo. A specific histidine residue in HSF1 DNA-binding domain was found to be involved this process. In addition, an acidic extracellular environment can launch the HSF1-participant pathway on the cell-line system which is involved in the intermolecular interaction between the protonated histidine and arginine.

EXPERIMENTAL

PLASMID CONSTRUCTION, POINT MUTATION, AND PROTEIN PURIFICATION

The bacterial expression vector pET21b (Novegen) containing the human HSF1 cDNA was previously constructed [Lu et al., 2008]. In this study, the mammalian expression vector pCDNA3 (Addgene) containing hHSF1 cDNA was also constructed with a C-terminal FLAG tag. The site-directed mutagenesis was performed to analyze the potential roles of the different histidines (His45, His63, His83, and His101), by substitution with alanine.

The bacterial plasmids containing HSF1 genes were respectively transformed into *Escherichia coli* BL21 (DE3). After the cell density (OD_{600}) was over 0.7 in LB medium, 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) was added into LB for further inducing the target proteins (20°C, 16 h) [Ahn et al., 2001]. For protein purification, the culture medium was centrifuged to collect the cells. An ice-cold cell lysis buffer (20 mM Tris–HCl (pH 8.0), 30 mM imidazole, 200 mM sodium chloride, 1 mM benzamidine, and 0.5 mM PMSF) was used to resuspend the cells for sonication. After centrifugation (13,000 rpm, 40 min at 4°C) of the crude lysate, the supernatant was purified by a HisTrap HP column and a mono Q HR 5/5 column (GE Healthcare) [Lu et al., 2008]. The proteins with high purity (> 90%) was confirmed by Coomassie blue staining, and stored at −80°C.

CROSS-LINKED TRIMERIZATION ASSAY (CTA) AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Protein samples were dialyzed for 6 h with a range of pH buffers (0.5× PBS buffer with 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and protease inhibitors; pH range from 7.4 to 5.2) at 4°C. For CTA, the samples were further incubated with 0.5 mM cross-linking chemical EGS (Ethylene glycol bis[succinimidylsuccinate]) solution for 10 min (Reaction was quenched with glycine), and mixed with 5 × non-reducing sample buffer [Lu et al., 2008]. Finally, the samples were boiled for 10 min at 95°C to completely denature the proteins and then loaded onto a no-reducing SDS–PAGE gel to determine the relative amounts of hHSF1 monomers, dimers, and trimers.

For DNA binding assay (EMSA), two synthetic complementary oligonucleotides were annealed to form a heat shock element (HSE) [Park et al., 1999b]. Then, T4 polynucleotide kinase labeled this HSE with [γ-^{32}P] ATP [Park et al., 1999a]. During protein-DNA binding treatment, the HSF1 proteins were incubated with ^{32}P-labeled HSE DNAs (~30,000 cpm [<1 ng]) (20 min, 25°C), and the reaction mixtures were then loaded into a 5% Tris–borate-EDTA native gel, and photo-developed by autoradiography.

MAMMALIAN CELL CULTURE AND IMMUNOBLOTTING

The HeLa cell line was obtained from ATCC. The immortalized mouse embryonic fibroblast (MEF) cell lines *hsf1*$^{+/+}$ and its knock-out, *hsf1*$^{-/-}$, were a kind gift from Prof. Yun-Sil Lee [Kim et al., 2009]. All cell lines were cultured in DMEM supplemented with 5% FBS and P/S at 37°C and 5% of CO_2.

For immunoblotting, the MEF *hsf1*$^{-/-}$ cells were transfected with pCDNA3 vectors containing hHSF1 or its histidine mutant DNA. After a 48 h cell culture, the medium was changed to a low pH (pH = 6) culture medium. After a further 6 h culture, the cells were washed twice in 1× PBS, and then resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, 1× protease inhibitor cocktail [Roche]) for 10 min. 10 μg of proteins extracted from the MEF cells were loaded on 12% SDS–PAGE, and transferred to a 0.45 mm nitrocellulose membrane (Fisher). The membrane was blocked in 1× PBS with 0.05% Tween 20 and 3% BSA for 1 h at room temperature and then incubated overnight at 4°C with the specific antibodies (HSF1, HSP70, HSP27 [Stressgen], and β-actin [Santa Cruz Biotech]). After washing three times with 1× PBS containing 0.5% BSA, the membranes were incubated with Alkaline Phosphatase-conjugated secondary goat-anti-mouse antibody (Sigma), washed three times, and developed using an enhanced chemiluminescence substrate system (Progmega).

CELL IMMUNOSTAINING AND IMAGING

For confocal microscopy, the HeLa cells transfected with C-terminal FLAG-tagged hHSF1 and its histidine mutant were grown in the lab-tek II chamber slides, fixed in a 4% formaldehyde solution (Pierce), and permeabilized for immune-fluorescence analysis as described previously [Debnath et al., 2003]. The primary antibodies were: HSF1 (Stressgen) and β-actin (Santa Cruz Biotech). The secondary antibodies were goat anti-mouse Alexa-488 (Invitrogen) antibodies. The images were obtained with a Zeiss LSM510 META confocal microscope. The double label images were acquired in multi-track mode with 4 times line averaging using a 63 × /1.4 oil DIC objective. The nuclei were stained with DAPI (Invitrogen). All microscopy processing and analysis was performed using Volocity high performance imaging software.

COMPUTATIONAL PREDICATION OF pKa VALUES AND Pymol

Several different software or web-serve programs were used to predict the pKa of the amino acid side chains residues. The default parameter sets were used in all cases. The protein structure files (PDB file) of five HSF-DBDs, which are NMR-solved human HSF1-DBD (PDB ID: 2LDU), were downloaded from NCBI. The calculations were performed on each individual structure in these computational programs.

The web-server, ***H*++3.0** (http://biophysics.cs.vt.edu/), uses the MEAD engine to solve the Poisson-Boltzmann (PB) equation by a combination of a 'smeared charge' concept [Anandakrishnan et al., 2012]. **PROPKA3.0** (http://enzyme.ucd.ie/cgi-bin/pKD/server_start.cgi) estimates the shift in pKa arising from hydrogen bonds, relative burial and coulombic interactions [Rostkowski et al., 2011]. The web interface, **Karlsberg+** (http://agknapp.chemie.fu-berlin.de/karlsberg/), is based on the numerical solutions of the linearized PB equation in combination with the structural relaxation of hydrogen and salt bridge [Kieseritzky and Knapp, 2008].

The rendering of all surfaces and models was completed in the Pymol Molecular Graphics System (Ver. 1.3, Schrodinger LLC).

RESULTS AND DISCUSSION

IN VITRO HSF1 ACTIVATION BY A SLIGHTLY ACIDIC pH

Commonly, the extracellular pH of the solid tumors is acidic, ranging from 6.2 to 7.0, and the pH in the blood plasma can decrease to below 7.0 during severe metabolic acidosis [Schornack and Gillies, 2003; McCarty and Whitaker, 2010]. In animal cells, the elevated environmental temperature strikes a significant decrease in pH (the pH_i decreases to 6.0) followed by HSF1 activation and HSP overexpression [Drummond et al., 1986; Zhong et al., 1999]. Recent studies reported the powerful multifaceted malignant roles of HSF1 in carcinogenesis [Dai et al., 2007]. To determine if the acidic pH within the physiological range affects HSF1 activation, a compelling experiment [Mosser et al., 1990] was performed. The cytoplasmic extracts from the human cervical cancer cell lines (HeLa cell) were incubated with various pH buffers (ranged from 5.0 to 7.8), and were examined the HSF1 activities (HSE binding activities). In results (7), the cytoplasmic extracts exhibited stable DNA (HSE)-binding activities (adjusted precisely to a pH from 6.4 to 5.8 with the maximal activity occurring at pH 6.0), suggesting that human HSF1 (hHSF1) can be activated directly under mild acidic pH condition. A similar phenomenon was also observed in-vitro in *Drosophila* HSF purified from the Sf9 expression system, and in mouse HSF1 synthesized from *E. coli* [Farkas et al., 1998; Zhong et al., 1999]. To confirm this above results, the recombinant hHSF1 proteins expressed from *E. coli* were purified, and treated with various pH buffers (similar to the above experiments with the pH from 7.6 to 5.2). The hHSF1-trimer (cross-linked by a chemical EGS) and DNA-binding (to HSE) (Fig. 1) could also be detected by western blot and EMSA assays in the samples treated with the buffers (pH 6.4–5.6), which concurs with previous EMSA results [Mosser et al., 1990; Farkas et al., 1998]. This suggests that a mild acidic environment (particularly the pH ~6.0 in hHSF1 and mHSF1) can directly trigger HSF1 activation (trimerization and DNA-binding) in vitro. It was shown that a more acidic solution (pH < 5.6) can truncate the HSF1 trimerization and DNA-binding (Fig. 1), which could be caused by the possible destruction of the native proteins under acute acidic pH condition.

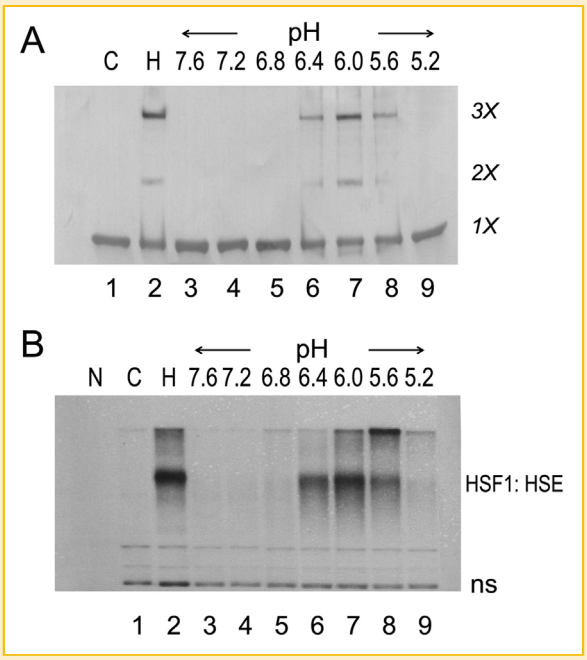

Fig. 1. In-vitro activation of human HSF1 (hHSF1) by low pH. Purified hHSF1 samples were dialyzed in 0.5× PBS at pH 5.2–7.6. A,B: Trimerization and DNA-binding assays were performed according to the detail protocols in the "Materials and methods" section. C, control; H, heat; N, no protein.

SLIGHTLY ACID ENVIRONMENT COULD PROTONATE HISTIDINE IMIDAZOLE RING, CAUSING THE HSF1 ACTIVATION

To our knowledge, a low pH can acidify (or protonate) the side chains of the specific amino acid (such as imidazole, aspartic acid). In histidine, the protonated form (conjugate acid) of the side-chain (imidazole ring) has a pKa of approximately 6.0, which means that, within the physiological-ranged pH decrease, relatively small shifts in pH could change its surface charge. Under a pH of 6 (histidine protonated), the imidazole ring bears two NH bonds and owns a positive charge by the Henderson–Hasselbalch equation. The positive charge is equally distributed between two neighbouring nitrogens with two resonance structures (Fig. 2A) [Li and Hong, 2011]. This new-generated positive charge could form various strong interactions (including intra- or extra-molecular), thus resulting in relevant physiological mechanism (i.e., protein catalysis, protein stability, protein–protein interaction, and protein–ligand interaction) in protein [Okada et al., 2001; Otomo et al., 2009; Van Gilst and Hudson, 1996].

Thirteen histidine residues are found within hHSF1 three pre-identified domains which are known as DNA-binding domain (DBD), trimerization domain (TD), and transcriptional activation domain (TAD). Five residues of histidine were distributed in the TAD of hHSF1. Previous experiments have showed that a deletion of TAD

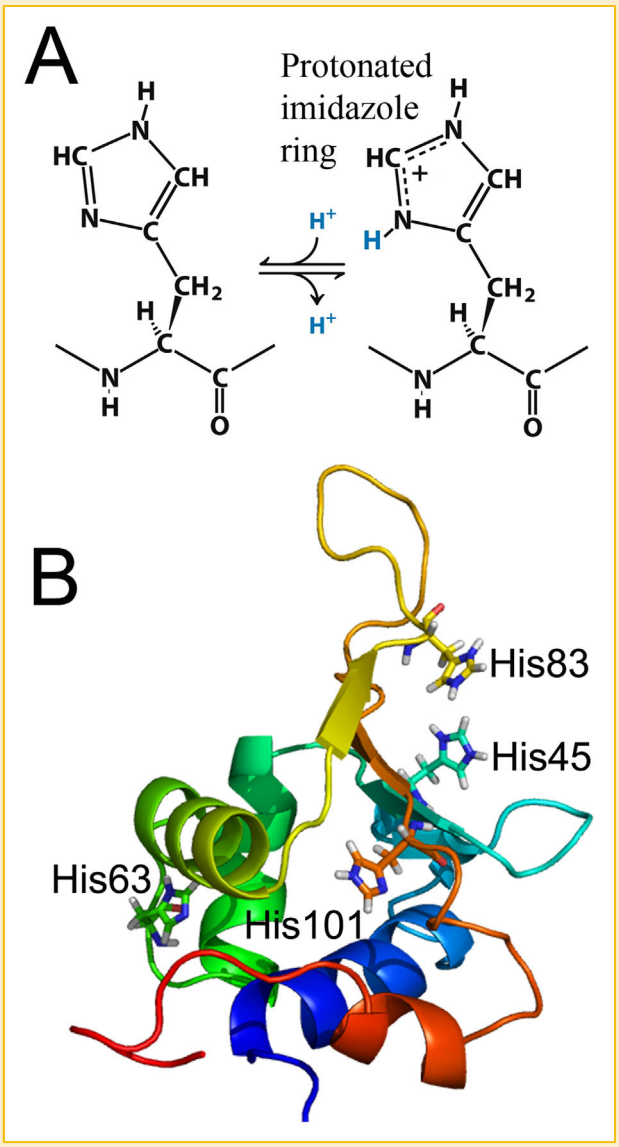

Fig. 2. A: The pH decrease within the physiological-range could protonate the imidazole ring of histidine. The imidazole ring in an isolate histidine could be ionized with an experimental pKa value of 6.0. Two practically equivalent share one position electron to form a resonance hybrid [Lu et al., 2013]. B: NMR structure of human HSF1 DNA-binding domain. The hHSF1-DBD was obtained from NCBI (PDB ID: 2LDU), and the positions of the His45, His63, His83, and His101 residues were labeled.

can facilitate the stress-induced hHSF1 trimerization and DNA-binding [Farkas et al., 1998], which suggests the dispensable role of this five histidines in the hHSF1-TAD. In the hHSF1-TD, another four histidines were hided into a triple-stranded, heptad-repeated coiled-coil motif [Peteranderl and Nelson, 1992; Peteranderl et al., 1999]. Sedimentation-equilibrium-based trimerization assays proved that under high pH (a pH of 8.8) condition, *Kluyveromyces lactis* HSF (KIHSF)-TD can still form a homotrimer in vitro after a treatment of cross-linking chemicals [Peteranderl and Nelson, 1992; Peteranderl et al., 1999], and a similar phenomenon was also detected in hHSF1 [Mosser et al., 1990]. Taken together, the histidine residue in TD and TAD seems not essential for HSF1-activation under a mild acidic-pH condition.

Thus, four residues (histidines at the position of 45, 63, 83, and 101) in the hHSF1-DBD were investigated to make certain whether a mild-acidic environment could protonize their imidazole rings, and thus activate hHSF1 to achieve the trimerization and DNA-binding capabilities (Fig. 2B). Although the standard pKa value of ionizable imidazole ring in an isolate histidine is tabulated in textbooks, a real pKa value is largely depended by the local microenvironment of a folded protein. Recently, the solution structure of hHSF1-DBD (PDB ID: 2LDU) was solved by NMR spectroscopy. By uploading this PDB file to three different web-based programs (PROPKA3.0, *H*++, and Karlsberg+), the pKa of the protonizable residues were predicted computationally [Tynan-Connolly and Nielsen, 2007; Rostkowski et al., 2011; Anandakrishnan et al., 2012]. Table I show that the predicted pKa values of His63 and His101 are less than the theoretical value (pKa 6.0) of an isolated histidine, whereas the residues His45 and His83 showed higher pKa values, suggesting that the imidazole rings of His45 and His83 could theoretically be ionized (or protonated) by a given low pH (such as pH$<$6.4). By screening other ionizable residues in hHSF1-DBD, most pKa values of the residue side chain were beyond the physiological pH range (Table S1), which suggests that only the histidine residue can be protonated. Furthermore, the open-source *H*++ web server could add the missing atoms and assign predefined partial charges to the uploaded PDB structures [Anandakrishnan et al., 2012]. At a given pH (pH = 6.4), the protonation states of ionizable groups can be described into a modified PDB file (Supporting File 1). The His45 and His83 residues, but not the His63 and His101 residues, were clearly shown in a protonated state in the new generated PDB structure (Fig. 2B).

To verify which histidine in hHSF1-DBD is involved in this mild acidification mediated activation, four histidines were point-mutated into alanines, respectively. The EMSA assay was analyzed after a treatment with a low pH (pH = 6.0) buffer, or heat shock (40°C) (Fig. 3). Pretreatment with low-pH or heat shock, the hHSF1 mutants (His45A and His101A) showed high DNA-binding activities, such as their wild-type, whereas the mutants of His63A and His83A did not reveal the pH- or heat-induced DNA-binding activities. Therefore, His45 and His101 are not essential for low pH-dependent DNA-binding.

In all known HSFs, the histidine at the position of 63 in hHSF1 is the most highly conserved one, which located in the "turn" region of a helix-turn-helix motif [Wu, 1995]. The structural analysis of a DBD-HSE complex in KIHSF clearly represent that the homolog of His63 (His242 residue in KIHSF) directly interacts with the major

TABLE I. Predicted pKa Values of Four Histidine in hHSF1-DBD

Histidine in hHSF1	PROPKA 3.0	*H*++3.0	Karlsberg+
His 36 (His 45)	6.76	9.4	13.6
His 54 (His 63)	6.03	4.3	0.9
His 74 (His 83)	7.57	7.1	7.3
His 92 (His 101)	5.00	4.0	−0.1

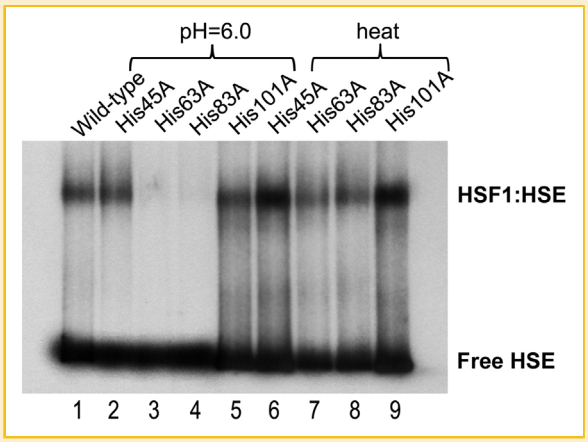

Fig. 3. Low-pH (pH = 6.0) or heat shock inducible DNA-binding activities. The protein samples from the wild-type and indicated histidine mutant forms of hHSF1 were assayed by EMSA using a ^{32}P-labeled HSE DNA fragment.

groove of HSE (especially in its phosphate backbone of the thymine base), together with the conserved residues (Asn, Ser, Gln, and Tyr) in the third α-helix of DBD (Supplementary Fig. S1A, Green Color) [Littlefield and Nelson, 1999]. Comparing to the no-DNA binding isoforms (PDB ID: 3HSF (Marine color, NMR-solved) and PDB ID: 1FBU (Magenta color, X-ray solved)), His242 is always exposed to the surface of KIHSF-DBD (Supplementary Fig. S1B) regardless of whether they are under stressful and non-stressful conditions. Meanwhile, a mutation of this histidine homolog to arginine (or alanine) in yeast *Saccharomyces cerevisiae* HSF (ScHSF) decreases both the constitutive and heat-induced activities [Bulman et al., 2001; Yamamoto and Sakurai, 2006]. However in the HSF-DBDs from the higher eukaryotic species (such as human and *Drosophila*), this residue homolog buries itself inside the DBDs (Supplementary Fig. S1C and S1D), in accordance with their intrinsic stress-sensing activation. These evidences revealed that the heat-induced location-change (or exposure) of this important histidine residue in higher eukaryotic organisms is responsible for heat-induced or constitutive HSE-binding, but not for low pH-induced HSF-activation. Therefore, the ionizable residue His83, which is located in the loop domain of hHSF1-DBD, is involved in the acidic-induced activation.

EFFECT OF THE ACIDIC EXTRACELLULAR PH ON HSF1-PARTICIPANT PATHWAY ON CELL-LINE SYSTEM

The intracellular pH can be affected by extracellular acidification, via intracellular sensors and/or by directly sensing the acidic extracellular pH. This occurs in MCF-7 cells cultured in a low pH medium (pH = 6.2) for 1 h, the internal pH decreased to 6.77 ± 0.01 [Balgi et al., 2011]. In this study, we examined if the acidic extracellular pH would enable the activation of the HSF1-partipant pathway by the involvement of histidine at the cell-line level. The mammalian expression vectors, pcDNA3, containing wild type hHSF1 and its mutant (His45A, His83A, and His101A) were transfected into the HSF1 knock out cell line (*hsf1*$^{-/-}$ MEF). After a 36 h culture, the MEF cells were then incubated with the low pH (pH = 6.0) medium that was pre-adjusted with 0.1 M hydrochloric acid in a DMEM medium before adding 10% FBS, and cultured for a further 4 h. The His45A and His101A mutant transfected cells produced the inducible HSP70 and HSP27 (Fig. 4), confirming the proposition that both histidines are not necessary for extracellular acidic pH-induced hHSF1 activation. Importantly, the His83A mutant showed a lack of HSP70 and HSP27 expression, which is similar to the mock-transfected MEF cells. This highlights the importance of His83 in low-pH-induced HSF1 activation.

Previous studies reported that in the absence of stress, the HSF isoforms are cytosolic, whereas they are translocated to the nucleus as a key step in the response to stress in many cell types [Akerfelt et al., 2010]. The hHSF1 His83A mutant was analyzed for its subcellular location under an extracellular acidic pH because this mutant derivative is defective in stress-induced trimerization and DNA binding in vitro, and the cells expressing this histidine mutants exhibit no stress-inducible HSP expression. HeLa cells were transfected transiently with the plasmids expressing the wild-type hHSF1 with C-terminal Flag tag, or the Flag epitope-tagged hHSF1 His83A mutant. Indirect immunofluorescence microscopy showed that under normal culture conditions, the wild-type hHSF1 was diffusely localized in the cells under steady-state conditions (Fig. 5A). In response to the acidic extracellular pH treatment, the Flag-epitope-tagged functional wild-type hHSF1 concentrated in the cell nuclei (Fig. 5B). In contrast, the hHSF1 His83A mutant did not show a cell nuclei concentration, which was similar to wild-type hHSF1 under non-stress conditions (Fig. 5A and C). Overall, the above experiments show that the His83 residue is essential for acidic-pH induced HSF1 activation in vitro and in vivo.

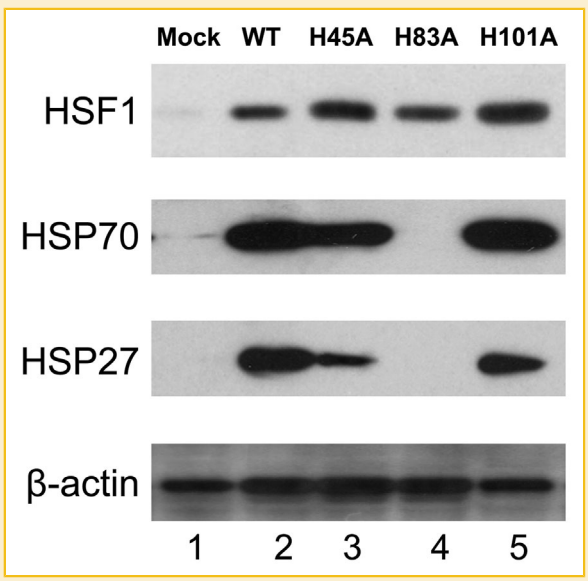

Fig. 4. Residue histidine in HSF1 confers acidic extracellular-induced activation of HSPs gene expression in *hsf1*$^{-/-}$ mouse embryonic fibroblast (MEF) cells. Immunoblot analyses for levels of HSF1, HSP70, HSP27 and β-actin were performed on the extracts prepared from the control or low-pH (pH at 6.0 for 4 h) *hsf1*$^{-/-}$ MEF cells transfected with pcDNA3 or a vector containing the indicated histidine mutant HSF isoforms.

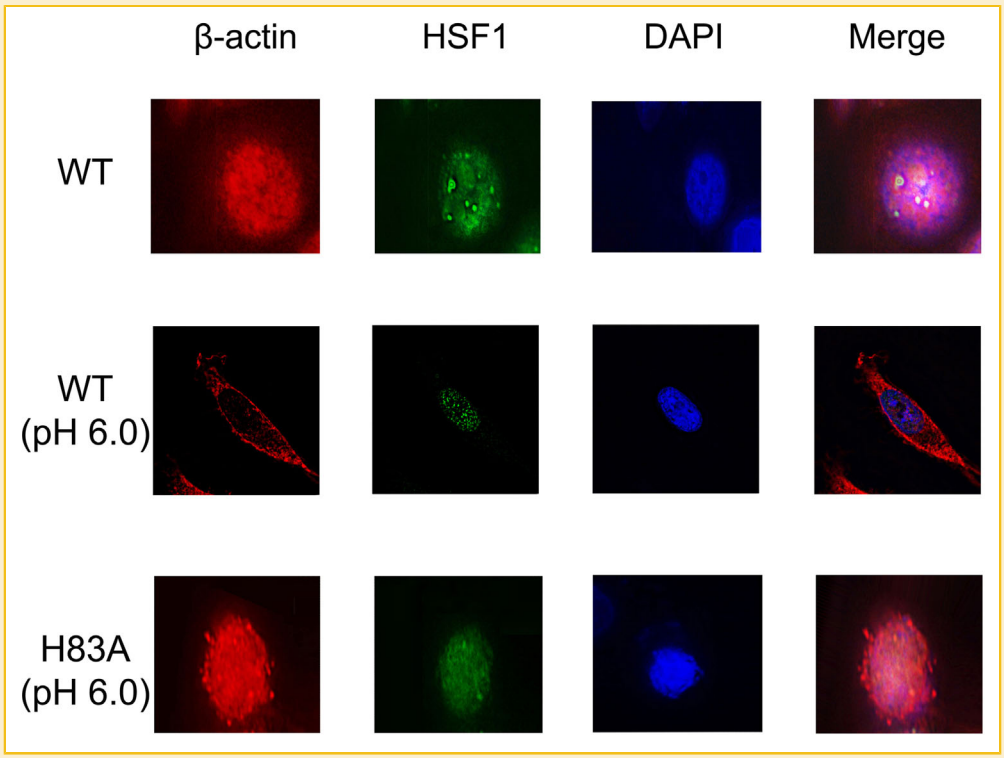

Fig. 5. His83 is essential for HSF1 low-pH induced nuclear translocation. Human HeLa cells were transfected transiently with the wild-type or histidine mutant of HSF1, which had been Flag-epitope-tagged. After 48 h, the cells were treated with low-pH medium for 4 h. HSF1 was detected by indirect immunofluorescence with the anti-Flag antibody and FITC-conjugated secondary antibody, with nuclear DNA staining with DAPI. All microscopy processing and analysis was performed using Volocity high performance imaging software.

PROTONATED His83 MAY BE INVOLVED IN ACIDIC-INDUCED HHSF1 ACTIVATION THROUGH A POSSIBLE INTERMOLECULAR INTERACTION WITH Arg79

His83 located in the most highly conserved region (a flexible loop motif between the third and fourth β-sheet) of hHSF1-DBD. Previous studies have proved that this supple region is critical for heat-inducible HSF activation both in mouse cell-line and in yeast *Saccharomyces cerevisiae* systems [Ahn et al., 2001; Cicero et al., 2001]. To further understand the roles of His83 in low-pH induced hHSF1-DNA-binding and trimerization, an on-line server (Cluspro 2.0 for protein–protein docking) [Comeau et al., 2004] and a PDB file of hHSF1-DBD structure (generated at pH = 6.0 from *H++* 3.0 program, Fig. 2B) were employed to generate a set of mimetic hHSF1-DBD homotrimers [Anandakrishnan et al., 2012]. From the output data, a reasonable structure might explain why His83 is involved in acidic-induced hHSF1-activation (Fig. 6B). In this computational structure, the hydrophobic interface of hHSF1-DBDs, which contain four β-sheets, could easily get close to form an internal hydrophobic core, together with their flexible, His83-containing loop motifs. In this situation, an extra-molecular interacting force between the protonated His83 and Arginine 79 (Arg79) was observed with the measured distances of 2.73, 4.11, and 4.38 Å (Fig. 6B). Previous study by molecular dynamic simulations [Heyda et al., 2010] proved that the side chain groups of protonated histidine and arginine has a propensity for forming like-charged contact pairs, if the distance is less than 6 Å. Furthermore in case of the interaction between insulin and its receptor, the dissociation binding constant decreased from 6.5 to 1.2 nM as the external pH was increased from 6.8 to 7.8. A histidine residue on the surface of the insulin receptor was found to interact with Arg22 in the insulin chain B via an intermolecular protonated histidine-arginine interaction [Halperin et al., 1987]. To confirm the hypothesis, low-pH induced trimerization and DNA-binding of the Arg79A mutant hHSF1 (Arg replaced to Ala) were analyzed in vitro (Fig. 6C and D). A lack of acidic pH- and heat- induced activities confirmed the importance of the Arg79 residue in both pH- or heat-induced hHSF1 activations. Furthermore, the mutation of Arg228, a homolog of Arg79, showed no growth on glucose at any temperature [Hubl et al., 1994]. In summary, two residues (Arg79 and prontonized His83) in hHSF1 could be induced to generate a like-charged contact pairs by a physiological-ranged acidic environment, which results in achieving hHSF1 trimerization and DNA-binding capability.

CONCLUSION

The heat shock response in eukaryotic organisms can be induced by a variety of environmental, chemical and pathophysiological conditions. Although these stressors are distinct in origin, the commonality of the stress-responsive HSF1 isoform is that they

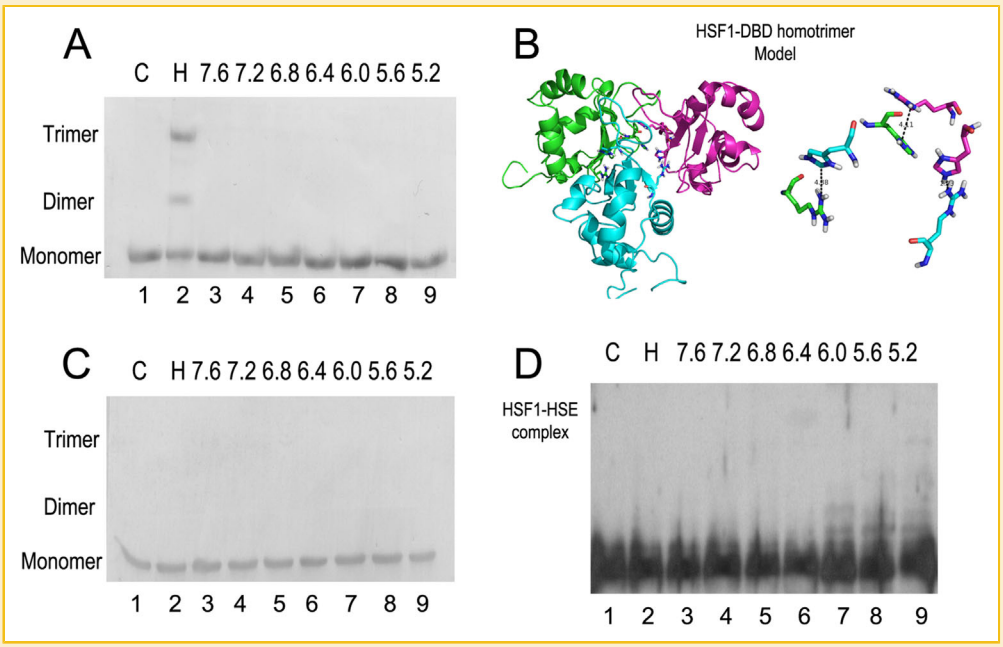

Fig. 6. Possible protonated histidine-participant intermolecular interaction in hHSF1-DBD trimer. A: Trimerization assay of the His83 mutant of hHSF1. B: Cluspro 2.0 generated hHSF1-DNA trimer. C,D: Trimerization and DNA-binding assays of the Arg79 mutant of hHSF1. The detail protocols were the same as those in Fig. 1. C, control; H, heat; N, no protein.

exhibit trimerization and DNA-binding activities. Nevertheless, the precise mechanisms by which this multitude of diverse signals is transmitted to or interpreted by HSF1 are unclear. This paper reports that the trimerization and DNA-binding of mammalian HSF1 is activated in vitro in a mild acidic pH environment. The protonated histidine residue (His83 in hHSF1) is essential for acidic pH induced HSF1-activation (including trimerization, DNA binding, nuclear accumulation and Hsps expression). In addition, a brand new intermolecular force (between Arg79 and His83) might be involved in this process to encourage HSF1 binding to HSE.

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